Perspective

Prodrugs of Phosphates and Phosphonates

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Introduction

Phosphonic acids are rarely found in drug candidate molecules, despite the potential of this functional group to provide unique binding interactions with a target enzyme or receptor. Similarly, organophosphates are rarely incorporated into drug candidates despite the common appearance of phosphates of nucleosides, proteins, lipids, and carbohydrates in nature. Much of the reluctance to use these groups stems from their high charge and consequently the inability of these molecules to achieve high concentrations at the target site due to poor oral bioavailability and/or cell penetration. Phosphates are further limited by their instability in most biological fluids because of the presence of phosphatases. Despite these difficulties, nucleoside phosphates and their phosphonate analogues have proven to be exceedingly important agents for anticancer and antiviral therapy, and phosphonate-containing drugs are increasingly being explored in other therapeutic areas.

In order to achieve oral bioavailability and intracellular delivery of phosphonates and nucleoside monophosphates, numerous prodrug strategies have been explored. For intracellular targets (the vast majority), success requires the prodrug not only to survive the gastrointestinal (GI) tract and be absorbed into the systemic circulation but also to remain intact in the systemic circulation long enough for it to be distributed into cells. The lack of adequate aqueous and metabolic stability greatly limits the effectiveness of many phosph(on)ate prodrug classes.

It has been over 10 years since the appearance of comprehensive reviews of this topic,[1,2] although a review of a more limited scope has appeared,[3] as well as reviews more specifically directed toward delivery of nucleosides and nucleotides[4–7] and a review focused on phosphate ester prodrugs.[8] The many developments in the field in the past decade warrant a comprehensive revisiting of the subject. In addition to chronicling the various types of prodrugs, this Perspective will offer a comparison of their real-world utility and will also highlight features beyond oral bioavailability and intracellular delivery, such as tissue-targeting.

Acyloxyalkyl Esters (Including Alkyloxycarbonyloxyalkyl Esters)

Whereas simple carboxylic acid esters are degraded rapidly in vivo by carboxylesterases, the corresponding simple alkyl esters of phosph(on)ates are typically metabolically stable and therefore are not useful as prodrugs. One of the most commonly used prodrug types for phosph(on)ates is the acyloxyalkyl ester.

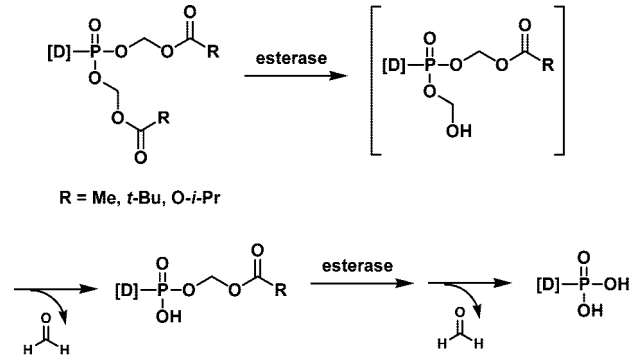

Figure 1. Activation of acyloxyalkyl ester prodrugs.

Enzymatic cleavage of the carboxylate or carbonate ester generates a transient hydroxymethyl intermediate, which rapidly loses formaldehyde to afford the acyloxyalkyl monoester (Figure 1). Hydrolysis of the monoester may proceed by a similar mechanism or may be catalyzed by a phosphodiesterase. This type of prodrug has been widely used for carboxylic acids, wherein the acetal carbon is often substituted by a lower alkyl group. When applied to phosphonates, this substitution leads to multiple stereoisomers, so examples of this type are relatively rare.

The esterase enzymes responsible for cleavage of these prodrugs, including carboxylesterases, paraoxonase, and cholinesterases,[9] exist in numerous tissues at high levels. Since esterases are present in the small intestine, prodrug cleavage prior to absorption can limit oral bioavailability. Once absorbed, these prodrugs are rapidly converted by esterases in blood and other tissues to the active drug. The chemical stability of these prodrugs can vary widely, and evaluating stability is an important aspect of optimizing prodrug properties. The solution degradation kinetics of members of this class have been described.[10]

The production of formaldehyde as a byproduct generated following cleavage of acyloxyalkyl esters has long been a potential safety concern, particularly for drugs intended for chronic use. This concern may be more perception than reality, given the substantial dietary intake of methanol and its predominant metabolism via formaldehyde to formate. It is noteworthy that two antiviral phosphonates are currently marketed that contain this type of prodrug, adefovir dipivoxil and tenofovir disoproxil. It is also worth noting that long-term exposure to pivalic acid is associated with carnitine depletion,[11] and thus higher doses of pivaloyloxymethyl (POM)[a] prodrugs could be of concern.

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[a] Abbreviations: POM, pivaloyloxymethyl; FdUMP, 2′-deoxy-5-fluo-

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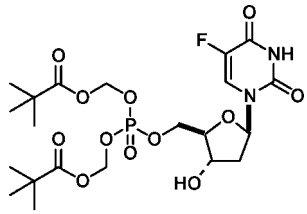


Figure 2. Structure of bis(POM) FdUMP.

Methods of synthesizing and initial hydrolysis studies on acyloxyalkyl prodrugs of nucleoside monophosphates were first reported in 1984 by Farquhar and co-workers, using phenyl phosphate as a model system.[12] This research group subsequently described the application to numerous nucleosides, including bis(pivaloyloxymethyl) prodrugs [bis(POM) prodrugs] of the monophosphates of 2′-deoxy-5-fluorouridine,[13] 2′,3′-dideoxyuridine,[14] thymidine,[15] and 3′-azidothymidine.[16] As an example, bis(POM) 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP) (Figure 2) displayed adequate chemical stability at acidic and neutral pH ($t_{1/2}$ > 40 h) and was converted to FdUMP in mouse plasma. Incubation with liver carboxylate esterases gave quantitative conversion to the mono(POM) derivative; the authors speculate that further conversion to FdUMP is catalyzed by plasma phosphodiesterases, although carboxylesterases may be responsible for this step as well. The agent was as effective as 5-fluorouracil at inhibiting the growth of Chinese hamster ovary (CHO) cells in vitro and at prolonging the life span of mice bearing P-388 leukemia. However, the authors note that the susceptibility of these prodrugs to rapid degradation in plasma will likely limit their systemic application in treating cell-based diseases.[13]

More recently, bis(POM) prodrugs have been utilized to enhance cellular delivery of the monophosphates of nucleosides 8-aza- and 8-bromo-2′-deoxyadenosine,[17] 2′-deoxy-4′-thioadenosine,[18] and 2′-azido-2′-deoxyuridine.[19] Application of acyloxyalkyl ester prodrugs to non-nucleoside monophosphate esters has also been reported. A recent example is the application to the *T. brucei* aldolase inhibitor 2-hydroxybenzaldehyde 5-phosphate, wherein the bis(POM) prodrug was shown to achieve modest whole-cell antiparasitic activity against *T. brucei* (IC_{50} = 20 μM).[20] Notably, these studies have been limited to in vitro assessments, likely because of the expectation of rapid degradation of these prodrugs in plasma.

Application to Phosphonates. The first report of the use of an acyloxyalkyl prodrug on a phosphonic acid was its application to the antibiotic fosfomycin in 1969.[21] Since then, it has seen continued use, typically being the first prodrug type tried when a prodrug of a phosphonic acid is desired. Two agents in this class are currently marketed for antiviral therapy, adefovir dipivoxil (for hepatitis B) and tenofovir disoproxil (for HIV) (Figure 3). The bis(POM) moiety of adefovir dipivoxil was selected from among a wide range of phosphonate prodrugs, including three acyloxyalkyl esters and the mono(POM) ester,

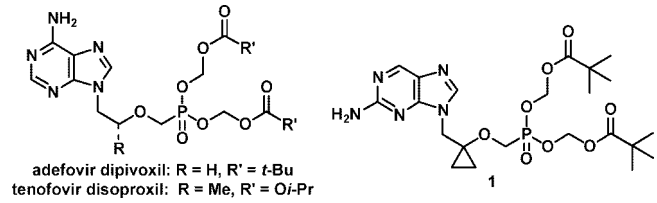


Figure 3. Structures of adefovir dipivoxil, tenofovir disoproxil, and prodrug **1**.

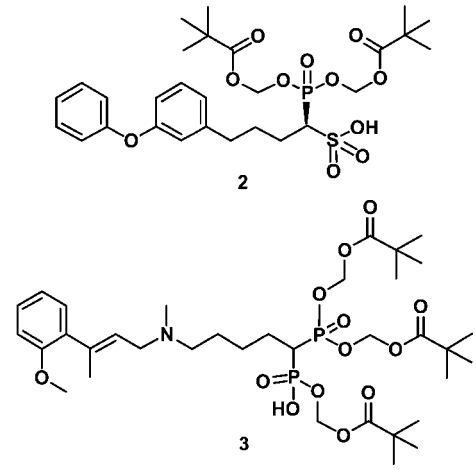


Figure 4. Structures of bis(POM) prodrugs **2** and **3**.

on the basis of oral bioavailability in rats.[22] In the case of tenofovir, the bis(isopropyloxycarbonyloxymethyl) prodrug (bis(POC)) was chosen on the basis of cellular activity in inhibiting HIV-1, chemical stability, and oral bioavailability.[23] More recently, the bis(POM) prodrug has been utilized in nucleotide analogue **1** (LB-80380) (Figure 3), a new agent for hepatitis B that is currently in phase 2 clinical trials.[24]

Outside the antiviral arena, the only agent of this class to have entered clinical development in recent years is the bis(pivaloyloxymethyl) squalene synthase inhibitor **2** (BMS-188494) (Figure 4), for hypercholesterolemia.[25] The pivaloyloxymethyl (POM) prodrug has been applied to another squalene synthase inhibitor **3** (ER-27856) (Figure 4), which has been studied for reducing cholesterol[26] as well as inhibiting the growth of *Trypanosoma cruzi*, the causative organism of Chagas' disease.[27] Interestingly, this latter agent employs three POM groups, whereas four would be required to fully mask the phosphonate charge. The existence of a single free phosphonic acid group undoubtedly benefits aqueous solubility while still allowing sufficient permeability for this highly lipophilic compound.

The bisphosphonates, synthetic analogues of endogenous pyrophosphate, are an important class of agents for treating osteoporosis and other diseases involving calcium metabolism. Prodrug approaches specific to the bisphosphonates have been reviewed.[28] The application of acyloxyalkyl esters to clodronate[29] and to etidronate[30] (Figure 5) was studied. In both cases the tris(POM) prodrug was named as the best candidate for enhancement of the oral bioavailability of the parent drug, based on lipophilicity, chemical stability, and rate of hydrolysis in serum and tissue homogenates. However, results of in vivo evaluation have not been reported.

Acyloxyalkyl prodrugs have also been utilized to improve the oral bioavailability of the antimalarial agent **4** (FR900098) (Figure 6). Initial investigations demonstrated that the bis(POM) derivative afforded improved efficacy relative to **4** in a mouse

rouridine 5′-monophosphate; F_2Pmp, phosphonodifluoromethylphenylalanine; SATE, *S*-acylthioethyl; ddUMP, 2′,3′-dideoxyuridine monophosphate; ddUTP, 2′,3′-dideoxyuridine triphosphate; DTE, dithioethanol; ddA, 2′,3′-dideoxyadenosine; AZT, azidothymidine; d4T, 2′,3′-dideoxydidehydrothymidine; ddC, 2′,3′-dideoxycytidine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; NEP, neutral endopeptidase; NMPA, naphthmethylphosphonic acid; BVDU, 5-bromovinyl-2′-deoxyuridine; PMEG, phosphonylmethoxyethylguanine; CYP, cytrochrome P_{450}; HCC, hepatocellular carcinoma; PTP1B, protein tyrosine phosphatase 1B; GI, gastrointestinal.

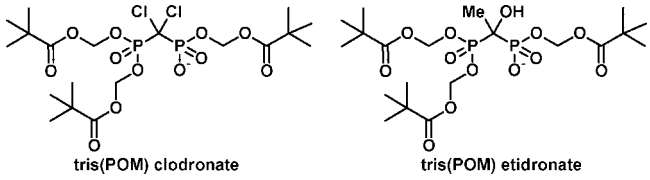


Figure 5. Structures of tris(POM) clodronate and tris(POM) etidronate.

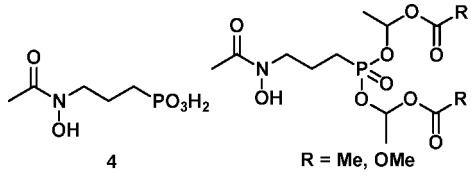


Figure 6. Compound **4** and prodrugs thereof.

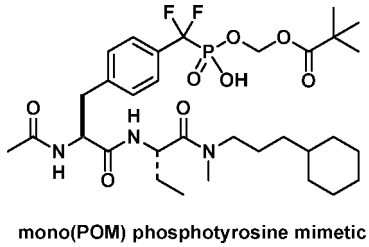


Figure 7. Structure of a mono(POM) phosphotyrosine mimetic.

model of antimalarial efficacy. In order to avoid the release of formaldehyde, prodrugs with substitution on the central methylene carbon were prepared, leading to identification of the bis(acetoxyethyl) prodrug as the most efficacious within this series.[31] Further studies examined the "carbonate ester" type (R = *O*-alkyl) and found the bis(methoxycarbonyloxyethyl) prodrug to be optimal within this class.[32] Other than the acknowledgment that mixtures were obtained, no mention is made in these reports of the issues surrounding developing complex diastereomer mixtures that result from substitution on the acetal carbon.

The POM prodrug has been applied to structures containing the phosphotyrosine mimetic phosphonodifluoromethylphenylalanine (F_2Pmp) (Figure 7) for the purpose of exploring structural and functional requirements of the phosphotyrosine-recognizing SH2 domains of kinases and phosphatases.[33] Interestingly, the authors were unable to prepare the bis(POM) derivative in this series, presumably either because of the poor nucleophilicity of the difluorophosphonate anion or instability of the product. However, unlike the mono(POM) prodrugs of the corresponding aryl phosphonates, the mono(POM) prodrugs of the F_2Pmp analogues were readily taken up into cells, allowing the functional activity of these agents to be evaluated. The oral bioavailability of the mono(POM) prodrugs was not assessed.

Phthalidyl esters have been explored as a particular type of acyloxyalkyl prodrug in which the carboxylate and aldehyde byproducts are covalently bound to each other, avoiding the formation of formaldehyde as a cleavage byproduct.[34] The trimethoxy phthalidyl ester shown in Figure 8 afforded high levels of the parent phosphonic acid in rat hepatocytes and displayed a half-life in plasma 17-fold longer than the bis(isobutyryloxymethyl) prodrug. However, the stereochemistry issues associated with substitution on the acetal carbon were not addressed.

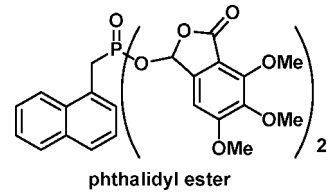


Figure 8. Phthalidyl ester prodrug of naphthylmethylphosphonic acid.

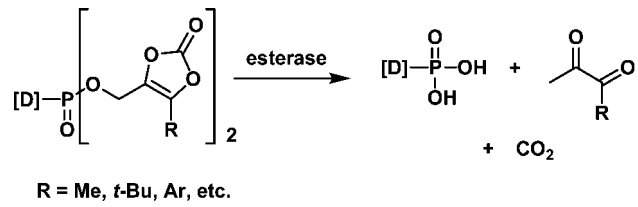


Figure 9. Activation of dioxolenone prodrugs.

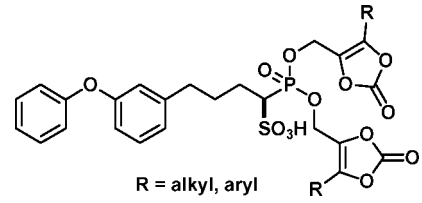


Figure 10. Dioxolenone prodrugs of **5**.

Dioxolenone Prodrugs. A prodrug type closely related to the acyloxyalkyl variety is the dioxolenone prodrug, which has been applied to carboxylic acids.[35] Like acyloxyalkyl esters, these prodrugs are rapidly cleaved in serum, presumably by carboxyesterases (Figure 9). Two important advantages for this prodrug type relative to acyloxyalkyl esters have been noted, namely, (1) the absence of a stereogenic center and (2) the production of a relatively nontoxic α-dicarbonyl byproduct rather than an aldehyde. The application of this prodrug moiety to phosphonates is discussed in an article whose primary thrust is a method for synthesis of substituted variants.[36] Also disclosed is the use of the method to prepare several prodrugs of the squalene synthase inhibitor **5** (BMS-187745) (Figure 10), although results of biological profiling of these prodrugs are not provided. Several examples of the application of this type of prodrug to phosphonates exist in the patent literature, but no studies have emerged in peer-reviewed journals.

S-Acylthioethyl (SATE) Esters

The *S*-acylthioethyl (SATE) ester prodrug has seen broad application to both phosphonates and phosphates. Like the aforementioned prodrugs, its cleavage is mediated by esterase enzymes widely distributed in blood and other tissues. Hydrolysis of the thioester yields an intermediate thioethyl ester, which decomposes with expulsion of ethylene sulfide (Figure 11). While the toxicity risk associated with this byproduct has largely limited the advancement of SATE prodrugs into development, they have been commonly used in in vitro studies for intracellular delivery of phosph(on)ates, particularly in the antiviral arena.

The first report of this type of prodrug described its application to the monophosphate of dideoxyuridine (ddUMP).[37] Whereas the triphosphate (ddUTP) is a potent inhibitor of HIV reverse transcriptase, the unphosphorylated nucleoside in this case lacks activity against HIV in a cellular assay because of its inefficient conversion to the monophosphate. The SATE

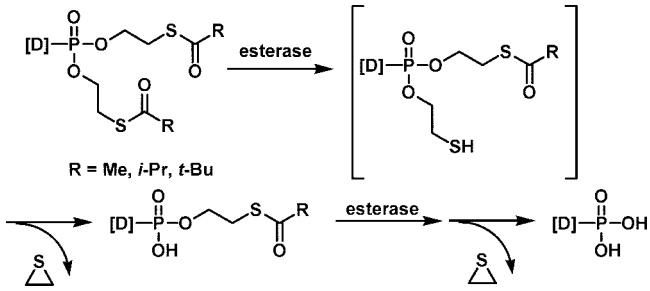


Figure 11. Activation of SATE esters.

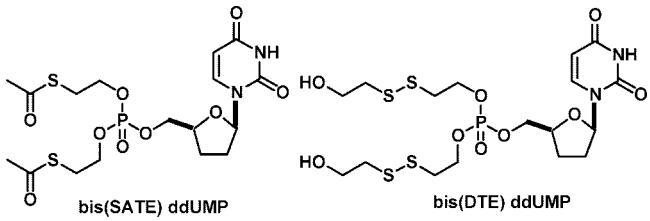


Figure 12. Structures of bis(SATE) and bis(DTE) esters of ddUMP.

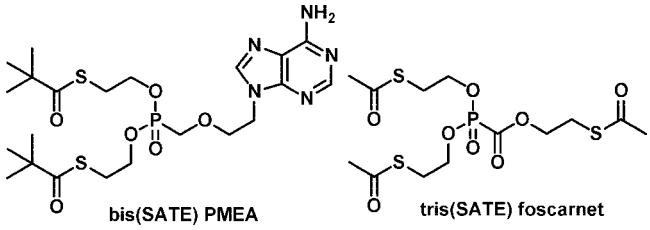


Figure 13. Structures of SATE esters of PMEA and foscarnet.

prodrug of the monophosphate (Figure 12) displays anti-HIV activity in two human T cell lines, showing the effectiveness of bypassing the first kinase[38] and that the prodrug is able to permeate cells. Also described in the same report is a related prodrug containing a dithioethanol (DTE) "trigger" (Figure 12), whose activation to the same mercaptoethyl intermediate is achieved by a reductase enzyme.[39]

Since this first report, SATE prodrugs have been widely applied to nucleoside monophosphates for the purpose of enhancing cellular activity, including application to the monophosphates of ddA,[40–42] AZT,[43–46] d4T,[47] acyclovir,[48–50] and ddC and 3TC.[51] Aspects of this work have been reviewed.[52] In addition, reports have appeared exploring the impact on prodrug properties of structural variations such as alkyl chain length,[53] thioalkyl chain variation,[54] and influence of C_α-substitution.[55]

The SATE prodrug approach has also been applied to phosphonates, with the SATE prodrug of PMEA being the first example described in the literature (Figure 13).[56] In this article, the authors report that the bis(*t*-Bu-SATE) prodrug displays antiviral activity comparable to that of bis(POM)-PMEA in cellular systems and that it possesses substantially greater stability in human gastric juice and human serum (serum $t_{1/2}$ of 4 h vs <5 min). These results suggested that the SATE prodrug might exhibit improved in vivo properties, but to date no further studies have appeared in the literature. The SATE prodrug of the antiviral agent phosphonoformate (foscarnet) has been studied (Figure 13); although antiviral activity was observed in cells, no phosphonoformate was observed in plasma following oral administration of tris(SATE) or bis(SATE) prodrugs to rats.[57] Prodrugs of this type have also been applied to enhancing cellular penetration of 5′-phosphonate analogues of adenosine[58]

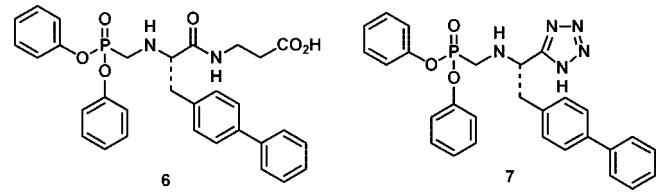


Figure 14. Structures of diphenyl esters **6** and **7**.

and small phosphopeptide inhibitors of the Grb2 SH2 domain.[59] The application of related *S*-acyl-3-thiopropyl (SATP) prodrugs has been studied with respect to foscarnet[60] and *N*-phosphonoacetyl-L-aspartate (PALA),[61] although they appear to degrade to the phosphonic acid more slowly than the corresponding SATE ester. Mixed prodrug approaches utilizing a SATE moiety along with a phosphonamide,[62] an aryl ester,[63] and a cyclic phosphate[64] have also been reported.

Aryl Esters

The first demonstration that a phosphonate diphenyl ester could achieve suitable prodrug properties (oral absorption and conversion to active drug in vivo) was in application to a neutral endopeptidase (NEP) inhibitor.[65] Whereas simple acyloxyalkyl ester derivatives were effective prodrugs, the authors were motivated to find a prodrug that did not release formaldehyde as a byproduct (α-methylene unsubstituted) or that did not contain unnecessary stereogenic centers (α-methylene substituted). Upon oral administration in rats, the simple diphenyl ester **6** (CGS 25462) (Figure 14) afforded peak levels of the active drug in plasma over 200-fold above the IC_{50}. Substitution by electron-donating groups afforded reduced levels of active drug in plasma, whereas substitution by electron-withdrawing groups led to chemically unstable compounds that hydrolyzed readily to the corresponding monoaryl phosphonates during purification. Aryl ester prodrugs of a related NEP inhibitor were also explored,[66] with similar findings, i.e., that the unsubstituted diphenyl ester **7** (CGS 26393) offered optimal plasma levels of active drug. Although the data have not been published, one can infer from other publications that **6** was advanced into human clinical trials.[67]

The hydrolysis of diphenyl ester **6** in aqueous buffers as well as plasma from various species has been studied.[68] Substantial differences in rates of cleavage between species (rat, dog, monkey, human), as well as the finding that thermal inactivation of plasma samples inhibited cleavage, led the authors to support an enzymatic cleavage mechanism. However, a dramatic difference in the rate of chemical hydrolysis of the monoester depending on the buffer used (pH 8.5; phosphate $t_{1/2} > 96$ h; carbonate $t_{1/2} \approx 0.5$ h) suggests the possibility that chemical hydrolysis is at least partially responsible for cleavage.

Other groups have reported results of application of diaryl esters of phosphonic acids. In a study of prodrugs of the close-in PMEA analogue 9-[2-(phosphonomethoxy)ethoxy]adenine,[69] the unsubstituted diphenyl ester was superior to substituted variants, giving 50% oral bioavailability of the active drug when administered as the hydrochloride salt. By contrast, the diphenyl ester of PMEA gave <3% oral bioavailability of active drug, whereas the bis(*o*-ethoxyphenyl) ester (Figure 15) achieved 40% oral bioavailability.[70] The latter authors report that these two prodrugs were stable in intestinal homogenate and plasma, whereas metabolism was observed in liver homogenate. In the case of the antimalarial **4**, the bis(4-methoxyphenyl) ester (Figure 15) was superior to its unsubstituted analogue, affording

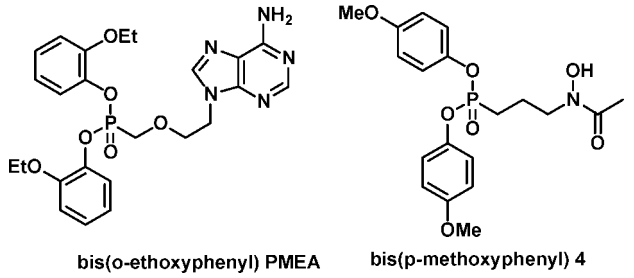


Figure 15. Structures of aryl esters of PMEA and **4**.

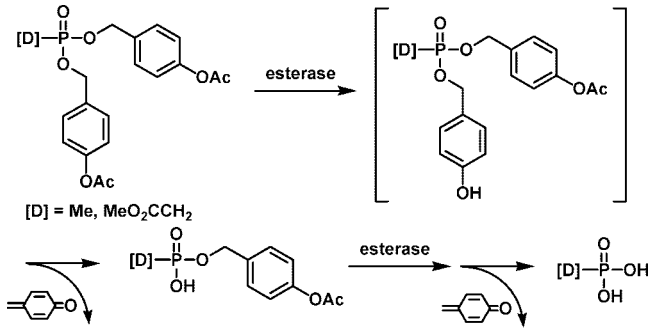


Figure 16. Activation of *p*-acetoxybenzyl prodrugs.

oral efficacy in a mouse parasitemia model comparable to that of **4** administered by the intraperitoneal route.[71]

Benzyl Esters

Simple unfunctionalized benzyl esters are not recognized by mammalian enzymes, but there are a few notable examples of phosphonate benzyl esters that are substituted in such a way as to render them useful as prodrugs. The strategy of the first researchers in this area was to use the benzene ring as a spacer between an esterase-labile acyl group and the phosphonate, with the rationale that maintaining a considerable distance between the negative charge of the monoanionic intermediate formed by hydrolysis of the first ester group and the remaining cleavable acyl group would render it a better esterase substrate. The intermediate 4-hydroxybenzyl ester was expected to undergo spontaneous fragmentation with loss of a quinone methide that presumably is hydrated to generate 4-hydroxymethylphenol (Figure 16). The initial application to phosphonoformate failed because of insufficient chemical stability, which was attributed to the adjacency of the carboxylate group to the phosphonate.[72] These researchers then explored application to the model system methyl phosphonate and the antiviral phosphonoacetate,[73] wherein sufficient chemical stability was demonstrated as well as cleavage by porcine liver carboxyesterase (PLCE). Although in vivo characterization is not described, the authors speculate that the observed acute toxicity of the 4-acetyloxybenzyl and 4-pivaloyloxybenzyl derivatives of methyl phosphonoacetate may be due to the reactive quinone methide generated by the fragmentation of the 4-hydroxybenzyl intermediate.

Further studies by the same group explored application to the 5′-monophosphate of azidothymidine (AZT),[74] 5,5′-nucleotide dimers containing AZT,[75] and phosphonate monoesters of phosphonoformate.[76] In addition, this type of prodrug has been studied as part of an investigation seeking orally bioavailable prodrugs of the PMEA analogue 9-[2-(phosphonomethoxy-)ethoxy]adenine, albeit with disappointing results.[69]

This group also studied alternative para substituents on the dibenzyl ester of phosphonoformate and noted that the bis(*p*-methoxybenzyl) prodrug was too unstable to be isolated.[77]

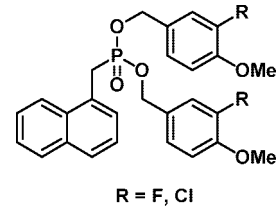


Figure 17. Structures of *p*-methoxybenzyl prodrugs of NMPA.

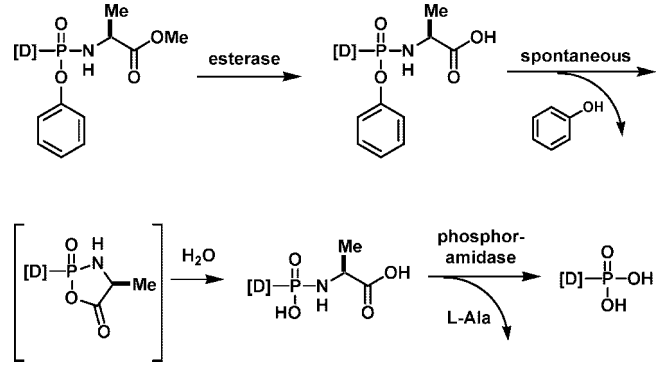


Figure 18. Activation of aryl phosphoramidate prodrugs.

Recently, investigation of bis(*p*-methoxybenzyl) prodrugs has been revisited, using naphthylmethylphosphonic acid (NMPA) as a model system (Figure 17).[78] Anticipating oxidative cleavage of the *O*-methyl group in the liver by cytochrome P_{450} enzymes, the rationale is provided that the reactive quinone methide intermediate will be trapped by glutathione, which exists in high concentration in liver cells. Whereas the simple bis(*p*-methoxybenzyl) prodrug exhibited insufficient chemical stability, the 3-chloro and 3-fluoro derivatives showed excellent stability in both buffer and rat plasma as well as efficient prodrug conversion to NMPA in rat hepatocytes and human liver microsomes.

Aryl Phosphoramidates and Phosphonamidates

The aryl phosphoramidate class of prodrugs (Figure 18) has been extensively investigated, particularly in application to nucleoside monophosphates.[79] In the prototypical structure, the two groups attached to the phosphorus atom are an α-amino acid ester and a phenol. The cleavage of this prodrug is initiated by an esterase enzyme; the newly revealed carboxylate is believed to undergo intramolecular cyclization with displacement of the phenol to form a short-lived five-membered ring intermediate, which is hydrolyzed to a phosphoramidic acid (Figure 18). Cleavage of the monoamidate to the active species may be catalyzed by a second (phosphoramidase) enzyme or may result from simple hydrolysis in a more acidic subcellular compartment (vide infra). The attachment of two different groups to the phosphorus atom creates stereoisomer issues because two diastereomers are generated using standard methods of synthesis. Moreover, the byproduct, phenol, is associated with toxicity at modest concentrations. Despite these potential concerns, numerous research groups have explored this prodrug type.

The first report of a prodrug of this structure, by McGuigan, et al.,[80] appeared shortly after a publication from the same group describing closely related prodrugs containing a trihaloethyl ester in place of the aryl ester.[81] This initial aryl phosphoramidate report, on a single derivative of AZT, was followed soon thereafter by a more extensive publication on AZT prodrugs exploring a range of substitutions on the prodrug moiety.[82] These

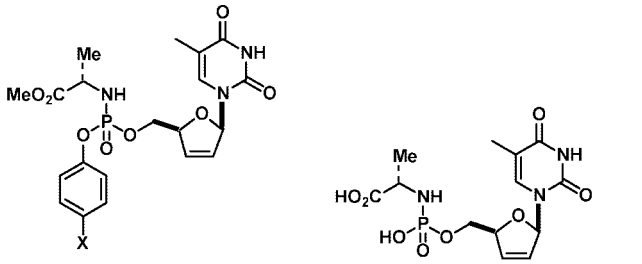


Figure 19. Structures of aryl phosphoramidates of d4T and the corresponding phosphoramidic acid intermediate.

studies, although limited to in vitro experiments, demonstrated the ability of this prodrug type to generate high intracellular levels of the monophosphate species and related metabolites. In particular, these prodrugs maintained activity in HIV-2-infected CEM cells lacking thymidine kinase (and thereby resistant to AZT). This group later reported extension of this approach to d4T and noted that cell cultures incubated with the prodrug **8** (So234) (Figure 19) contained levels of the intermediate alaninyl phosphoramidic acid metabolite 10- to 15-fold higher than those of d4T triphosphate.[83] Further, the role of this intermediate as an intra- and/or extracellular depot form of d4T-monophosphate was proposed.[84] The rat liver phosphoramidase enzyme believed to be responsible for conversion of this phosphoramidic acid to the nucleoside monophosphate was shown to be distinct from creatine kinase, alkaline phosphatase, and phosphodiesterase.[85]

Structure–activity relationships in the d4T series were established by exploring various amino acids and demonstrated that although L-alanine is preferred,[83] α,α-dimethylglycine is also converted,[86] as well as certain other unnatural amino acids.[87] Substitution on the aryl moiety was also investigated, and the researchers found that more lipophilic analogues displayed superior activity in a cell-based assay despite a similar rate of esterase-mediated degradation, presumably as a result of enhanced cellular permeation.[88,89] This latter finding was followed by intensive study by other researchers of the *p*-bromophenyl analogue of **8**, stampidine (Figure 19), which has been the subject of numerous reports of preclinical studies, including pharmacokinetics and metabolism,[90,91] toxicity,[92] anti-HIV efficacy,[92,93] and large-scale GMP synthesis and formulation.[94] Stampidine is a mixture of two diastereomers, which have been separated and shown to have equivalent anti-HIV activity.[95] Despite the thorough investigation of the properties of stampidine, no reports of its advancement to human clinical trials have appeared. The pharmacokinetic studies highlight the importance of the alaninylphosphoramidic acid intermediate as a long-lived active metabolite.

Later studies by McGuigan et al. explored application of the aryl phosphoramidate pronucleotide (ProTide) technology to abacavir (Figure 20),[96] carbocyclic adenosine derivatives,[97] and 4′-azidouridine.[98] In vitro studies by other researchers have investigated the application of this prodrug type to 8-aza-isoddA and isoddA for HIV,[99,100] methylenecyclopropane analogues of nucleosides for cytomegalovirus,[101,102] dioxolane−thymine analogues for HIV,[103] and 6-hydrazinopurine 2′-methylribonucleosides for hepatitis C.[64] Of these investigations, the only one to report in vivo characterization is the application to abacavir, itself a prodrug of the HIV reverse transcriptase inhibitor carbovir, wherein an aryl phosphoramidate prodrug was administered to cynomolgus monkeys.[96] Upon iv administration, the prodrug was cleared extremely rapidly from plasma with an elimination half-life of 7 min; the most abundant metabolite was the alaninyl phosphoramidic acid intermediate. When administered orally, the most abundant metabolite in plasma was abacavir, with the second most abundant being the alaninylphosphoramidic acid intermediate.

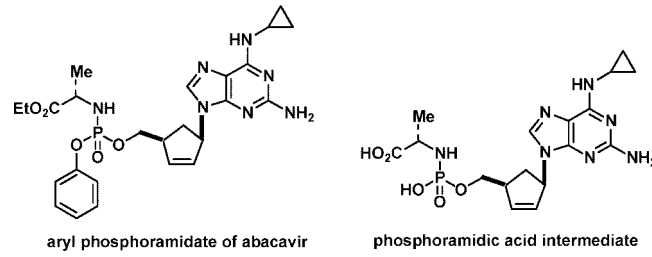


Figure 20. Structures of an aryl phosphoramidate of abacavir and the corresponding phosphoramidic acid intermediate.

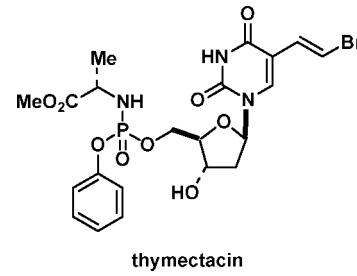


Figure 21. Structure of aryl phosphoramidate thymectacin.

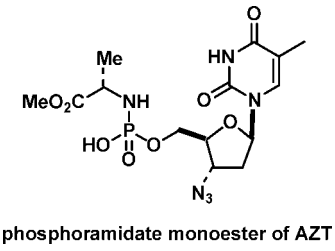


Figure 22. Phosphoramidate monoester of AZT.

Despite the numerous applications of aryl phosphoramidate prodrugs to nucleoside monophosphates, only one has been reported to have been advanced to human clinical trials. Thymectacin is an aryl phosphoramidate prodrug of the monophosphate of the anti-VZV agent 5-bromovinyl-2′-deoxyuridine (BVDU, brivudin) (Figure 21). It was originally reported by McGuigan et al. to be 5- to 25-fold *less* potent than BVDU against VZV in tissue culture.[104] However, other researchers have reported selective toxicity to tumor cells expressing elevated levels of thymidylate synthase (TS)[105] and have advanced this agent to human clinical trials as a treatment for colon cancer,[106] apparently as a mixture of diastereomers.[107]

Phosphoramidate Monoesters. Inspired by the above studies of aryl phosphoramidates, Wagner et al. have explored the utility of phosphoramidate monoesters, i.e., lacking the aryl ester (Figure 22).[108] Not surprisingly, the corresponding prodrug of AZT monophosphate displays poor oral bioavailability and undergoes substantial degradation to AZT in the GI tract.[109,110] Nevertheless, for intravenous administration, this type of prodrug may represent a useful alternative method of delivering a nucleoside monophosphate (although it must be kept in mind that cellular permeation will likely be slow because of its anionic nature). Activation proceeds directly to the monophosphate via

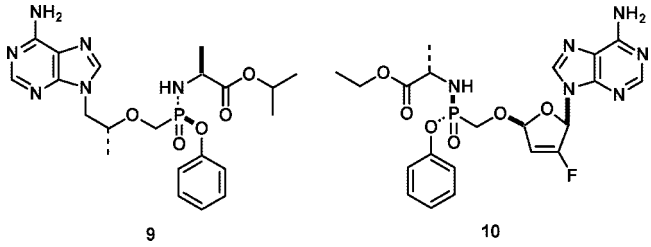


Figure 23. Structures of aryl phosphonamidates **9** and **10**.

the action of a phosphoramidase, identified as Hint-1, without the intermediate action of a carboxylesterase.[111]

Application to Phosphonates (Aryl Phosphonamidates). Compared with nucleoside monophosphates, there have been relatively few reports of the use of this type of prodrug with phosphonates. In 2001, McGuigan et al. reported the synthesis and in vitro evaluation of several such prodrugs of the antivirals PMEA (adefovir) and PMPA (tenofovir).[112] Relationships between structure and antiviral activity were similar to those observed in their earlier investigations with nucleoside monophosphates, with the L-alaninyl methyl ester affording the best activity. More recently, researchers from Gilead Sciences have reported investigation of **9** (GS-7340), a single diastereomer aryl phosphonamidate prodrug of tenofovir containing an L-alanine isopropyl ester (Figure 23).[113] These researchers noted that the L-alanyl diastereomer containing the opposite configuration at phosphorus displayed anti-HIV activity in vitro 12-fold less potent than **9** and therefore developed methods for large-scale isolation of **9** from a diastereomeric mixture using simulated moving bed (SMB) chromatography.[114,115] A remarkable enhancement of distribution and accumulation in lymphatic tissue was observed relative to tenofovir disoproxil (an acyloxyalkyl ester), which was attributed to increased metabolic activity in these tissues combined with the long intracellular half-life of tenofovir and its mono- and diphosphate metabolites.[113] Oral bioavailability of 17% (relative to an intravenous dose of tenofovir) was observed in the dog, and this agent was advanced to human clinical trials (which were subsequently discontinued).[116] The hydrolase responsible for the initial step (ester cleavage) in the activation of both **9** and the cyclic nucleotide analogue **10** (GS-9131) (Figure 23) in human peripheral blood mononuclear cells was identified as lysosomal carboxypeptidase A (cathepsin A).[117] The phosphonamidase responsible for conversion of the intermediate phosphonamidic acid derived from **9** to the active agent has not been characterized; however, these researchers note that simple chemical hydrolysis of this intermediate is extremely fast at low pH and that therefore spontaneous hydrolysis in a cellular compartment with low pH cannot be ruled out.[113]

Phosphoric and Phosphonic Diamide Prodrugs

Diamide prodrugs have two distinct advantages relative to aryl phosphoramidate prodrugs. First, the byproducts of cleavage consist exclusively of nontoxic amino acids. Second, since two identical groups are attached to the phosphorus atom, it is not a stereogenic center and therefore there are no issues associated with generation of diastereomers upon prodrug formation. The initial step of prodrug hydrolysis, an esterase-mediated carboxyl ester hydrolysis, is similar to that of aryl phosphoramidates. The initial monoacid hydrolysis product is believed to spontaneously cyclize to form a five-membered ring intermediate, which further hydrolyzes to give an intermediate monoamidate species (Figure 24). While this monoamidate is structurally identical

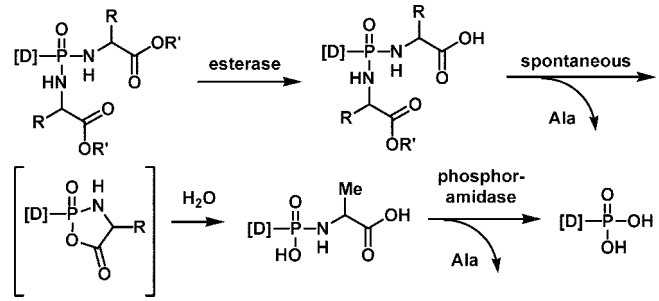


Figure 24. Activation of phosphonic diamide prodrugs.

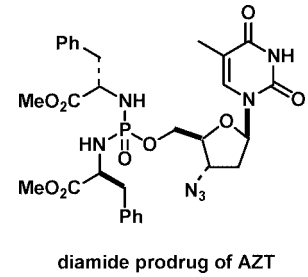


Figure 25. Diamide prodrug of AZT.

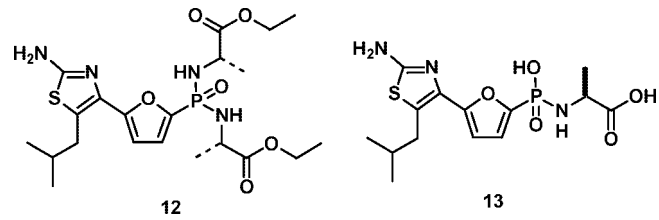


Figure 26. Structures of phosphonic diamide **12** and phosphonamidic acid intermediate **13**.

to that derived from an aryl phosphoramidate prodrug, the enzyme(s) responsible for cleavage to the active agent may vary with the particular drug (vide infra).

Although diamides have existed in the literature for over 15 years, they have only recently emerged as an important class of prodrugs (vide infra). McGuigan et al. reported the first use of a diamide prodrug to mask a nucleoside monophosphate, in application to AZT (Figure 25).[118] Interestingly, no further reports ensued seeking to explore and optimize the utility of this class of nucleoside monophosphate prodrugs from these or other researchers. The initial application of amino acid ester diamide prodrugs to a phosphonate-containing drug offered no hint of the ultimate promise of this class. The agent in this case was the PMEA analogue 9-[2-(phosphonomethoxy)ethoxy]adenine. Only one diamide prodrug was prepared (in the context of numerous other varieties), derived from 2 mol of glycine methyl ester; this prodrug failed to afford any detectable blood levels of the active drug following oral administration to mice.[69]

Despite these disappointing earlier results and the slow evolution of this class, the utility of phosphonic diamide prodrugs was resoundingly demonstrated with the emergence of a report describing application to the fructose-1,6-bisphosphatase inhibitor **11** (MB05032). Prodrug **12** (MB06322, CS-917) (Figure 26), derived from **11** and 2 mol of L-alanine ethyl ester, was advanced as a clinical candidate for the treatment of type 2 diabetes. The selection of this particular prodrug followed the preparation and extensive evaluation of numerous prodrug varieties, with the aim of finding a prodrug with improved oral bioavailability, good aqueous stability, and no risk of prodrug byproduct-related toxicity.[119] Compound **12** exhibits excellent

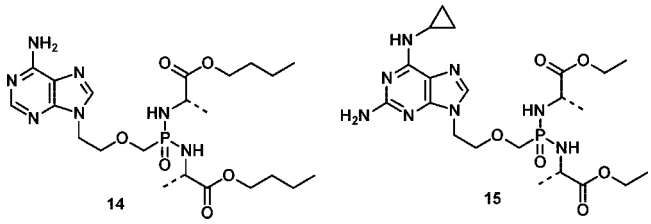


Figure 27. Structures of phosphonic diamides **14** and **15**.

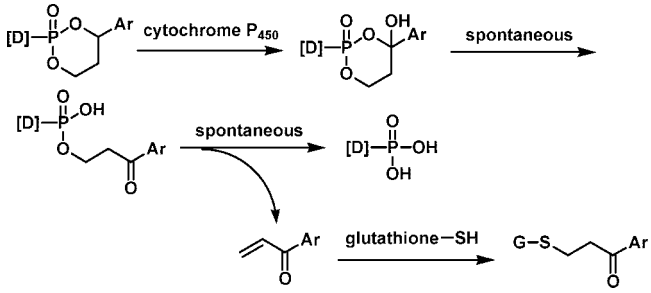


Figure 28. Activation of HepDirect prodrugs.

aqueous stability (t_{90} > 7 days at pH 3.0–7.4), yet is rapidly converted both in vitro (hepatocytes) and in vivo to **11**. The alanylphosphonamidic acid intermediate **13** is also observed, consistent with the proposed mechanism of prodrug activation. Compound **12** displays oral bioavailability in the rat of >20%, representing the first successful report of achievement of oral absorption with a diamide prodrug. This agent has been advanced to human clinical trials, wherein dose-linear absorption and efficient conversion to the active drug **11** were observed.[120] A report detailing the development of phosphonic diamide prodrugs has recently appeared.[121]

The phosphoramidase enzyme responsible for cleavage of the phosphonamidic acid intermediate **13** has been isolated and characterized.[122] On the basis of its molecular weight and preferred substrates, this enzyme was shown to be distinct from the phosphoramidase that catalyzes the final step in the conversion of aryl phosphoramidates of AZT and d4T.[85] Furthermore, previously reported aryl phosphoramidates of AZT monophosphate and PMEA were not hydrolyzed by this phosphoramidase preparation.

Researchers at Gilead Sciences have also explored the utility of diamide prodrugs. A study seeking inhibitors of orthopoxvirus replication demonstrated that **14** (GS-8357) (Figure 27), a PMEA prodrug derived from 2 mol of L-alanine *n*-butyl ester, was as effective as the bis-POM prodrug in cellular antiviral assays, indicating effective cellular permeation and intracellular prodrug cleavage.[123] More recently, the PMEG (phosphonomethoxyethylguanine) prodrug **15** (GS-9219) (Figure 27) has been reported to selectively deliver the active agent to lymphoid cells. In this case, cleavage of the phosphonic diamide is followed by deamination and phosphorylation to afford the active metabolite PMEG diphosphate.[124]

Cyclic 1-Aryl-1,3-propanyl Ester (HepDirect) Prodrugs

Because most nucleoside monophosphate prodrugs inefficiently deliver the monophosphate (or phosphonate analogue) to the target cell because of premature prodrug cleavage in other tissues, particularly the bloodstream, HepDirect prodrugs were designed specifically to be activated within the liver to treat liver-based diseases.[125] Key characteristics that were sought included (1) high oral absorption, (2) rapid activation by a single enzyme expressed predominantly in the liver, (3) good stability

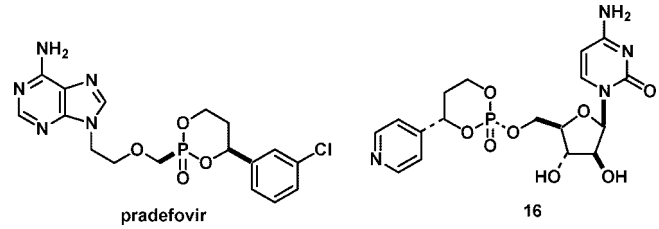


Figure 29. Structures of pradefovir and **16**.

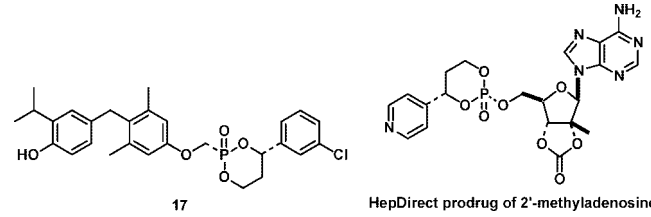


Figure 30. Structures of **17** and a HepDirect prodrug of 2′-methyladenosine.

in aqueous solutions, blood, and nonhepatic tissues, and (4) no byproduct-related toxicity. Activation is achieved via cytochrome P_{450}-mediated oxidation of a benzylic carbon atom adjacent to a phosph(on)ate oxygen, generating a cyclic hemiketal that undergoes spontaneous ring opening followed by β-elimination to afford the free phosph(on)ate (Figure 28). The byproduct of this elimination, an aryl vinyl ketone, would appear to raise toxicity concerns because it is highly electrophilic. However, since cells expressing cytochrome P_{450} enzymes also express high levels of glutathione (>5 mM) as a natural defense mechanism against damage from free radicals generated during CYP-mediated oxidations, the aryl vinyl ketone was expected to be rapidly and quantitatively captured as the glutathione conjugate.[125] These predictions were supported by studies evaluating the potential for byproduct toxicity in rat hepatocytes and in mice.[126] Methods for synthesis of HepDirect prodrugs with high diastereoselectivity have been reported.[127]

Three agents utilizing HepDirect prodrugs have been advanced to human clinical trials: pradefovir, **16** (MB07133), and **17** (MB07811) (Figures 29 and 30). Pradefovir (earlier known as remofovir) is a prodrug of PMEA (adefovir) in development for therapy of hepatitis B infection.[128] Studies using radiolabeled materials demonstrated that delivery using the HepDirect prodrug compared with adefovir dipivoxil achieves increases in liver/kidney and liver/intestine PMEA-derived metabolite exposure ratios of 12- and 84-fold, respectively.[126] Because adefovir dipivoxil is approved for use at a suboptimal dose for efficacy because of kidney toxicity,[129] pradefovir was advanced into human clinical trials with the aim of achieving superior efficacy with similar or better safety. Its pharmacokinetics in humans have been reported,[130] as well as in rats and cynomolgus monkeys.[131] In a phase 2 clinical trial at doses of 10–30 mg, antiviral efficacy superior to adefovir dipivoxil was achieved along with lower plasma exposure to adefovir.[132] A review surrounding pradefovir has recently appeared,[133] and extension of HepDirect prodrug technology to the hepatitis B antiviral agent lamivudine has been described.[134] The use of HepDirect prodrugs for targeting nucleotide-based antiviral drugs to the liver has been reviewed.[135]

The liver-targeting feature of HepDirect prodrugs has also been applied in an oncology context for treatment of hepatocellular carcinoma (HCC). An investigation of the synthesis and evaluation of HepDirect prodrugs of the monophosphate of the cytotoxic nucleoside cytarabine (araC) found that the 4-pyridyl

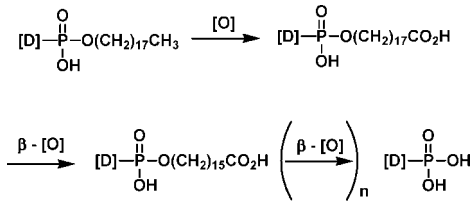


Figure 31. Activation of lipid ester prodrugs.

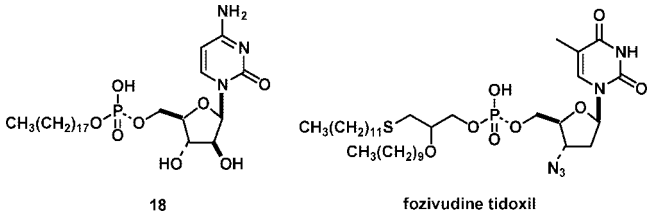


Figure 32. Structures of lipid esters **18** and fozivudine tidoxil.

derivative **16** displayed a superior profile with respect to activation, stability, and solubility for parenteral administration.[136] Studies in mice showed a >120-fold and >28-fold increase in liver/plasma and liver/bone marrow ratios relative to administration of araC. Initial evaluation in a phase 1/2 clinical trial in HCC patients demonstrated good tolerability, dose-linear pharmacokinetics, and preliminary evidence of efficacy.[137]

The use of a HepDirect prodrug to achieve liver targeting of a thyroid receptor (TR) agonist for lowering serum cholesterol has been reported. The 3-chlorophenyl prodrug **17** displays potent efficacy in a rat model of hypercholesterolemia without concomitant side effects associated with non-liver-selective TR agonists.[138,139] Compound **17** has entered clinical trials in humans.[140] In addition, the utility of HepDirect prodrugs in achieving dramatically improved triphosphate liver levels from the anti-HCV nucleoside 2′-methyladenosine has been reported.[141]

Lipid Esters

The concept of using lipophilic monoesters of a nucleoside monophosphate was first proposed as a means of improving the properties of the antileukemia agent cytarabine (araC) with the rationale that such esters would avoid metabolic degradation by cytidine deaminase, circumvent tumor resistance associated with decreased deoxycytidine kinase activity via kinase bypass, and achieve sufficient intracellular levels due to the partial masking of the phosphate charge and increased lipophilicity.[142–145] Cleavage of the prodrug moiety was expected by initial researchers to occur via the action of phosphodiesterase; however, it was subsequently shown that metabolism to the active drug is far more complicated, proceeding by successive two-carbon degradation via β-oxidation in peroxisomes (Figure 31).[146]

The initial investigations in this class were followed by entry into clinical development of the stearyl ester of cytarabine (**18**, cytarabine ocfosfate, YNK01) (Figure 32), which offered potential for oral administration combined with substantial extension of the half-life of cytarabine.[147–149] It was demonstrated in a phase 1/2 study that 15.8% of the total dose was absorbed and metabolized to araC and araU.[150] Compound **18** was first approved in 1992 for the treatment of leukemia.[151] In a similar vein, fozivudine tidoxil (Figure 32), a glycerolipid ester prodrug of AZT, is being advanced as a once-a-day alternative to AZT for therapy of HIV infection.[152]

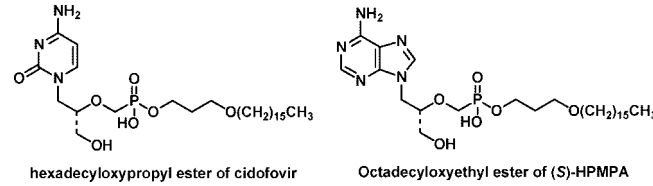


Figure 33. Lipid ester prodrugs of cidofovir and (*S*)-HPMPA.

More recently, numerous reports have emerged from the group of Hostetler et al. on lipid esters of nucleoside monophosphates and nucleotide phosphonates. These agents were initially designed to achieve maximal incorporation into liposomes, with the rationale that this would enhance uptake into macrophages and result in greater efficacy against HIV.[153] These initial investigations based on AZT and other nucleosides determined that such derivatives achieve efficient incorporation into cells and can afford superior activity in cases where kinase bypass is effective, and subsequent studies on acyclovir demonstrated the potential for high oral bioavailability.[154] Cellular studies using radiolabeled cidofovir demonstrated that the hexadecyloxypropyl prodrug (Figure 33) achieves a >100-fold increase in intracellular levels of cidofovir diphosphate relative to those attained with the unmodified nucleotide,[155] leading to enhanced antiviral activity against orthopoxvirus,[156] cytomegalovirus,[157,158] herpesvirus[157] and adenovirus.[159] More recently, these investigations have been extended to prodrugs of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine ((*S*)-HPMPA) (Figure 33).[160,161]

Despite the notable success of cytarabine ocfosfate, the application of these prodrugs has largely been limited to a small circle of researchers, perhaps because of the undesirable physical properties (lack of crystallinity, in particular) that the lipid moiety undoubtedly imparts. Applications of these prodrugs for the treatment of cancer have recently been reviewed.[162]

Nitrofuranylmethyl Amidates

Nitrofuranylmethyl amidates have been explored as phosphate and phosphonate prodrugs. With the rationale that the intermediate phosphoramidate derived from other phosphoramidate esters may degrade directly to the nucleoside rather than its monophosphate, Borch et al. synthesized and evaluated derivatives of FdUMP designed to spontaneously degrade to the monophosphate.[163] The nitrofuranylmethyl group is anticipated to be reduced intracellularly by a reductase enzyme, which spontaneously fragments to produce an intermediate chlorobutyl phosphoramidate (Figure 34).[164] The neighboring negative charge increases the nucleophilicity of the nitrogen atom, causing spontaneous cyclization to form an *N*-phosphoryltrialkylammonium species that is further hydrolyzed to afford the monophosphate.

The intracellular delivery of FdUMP (Figure 35) was demonstrated by the prodrug having comparable activity in wild type and thymidine kinase deficient cells.[163] These findings were extended to delivery of cytarabine monophosphate,[165] as well as to farnesyl monophosphate[166] and a phosphonate inhibitor of PTP1B (Figure 35).[167] Reports of in vivo application of these prodrugs have not appeared.

Cyclosaligenyl (cycloSal) Prodrugs

Although most prodrug practitioners utilize enzymes present in vivo to accomplish the release of the active drug, some have sought metastable prodrugs that are designed to achieve their desired aims by chemically degrading at an optimal rate. This

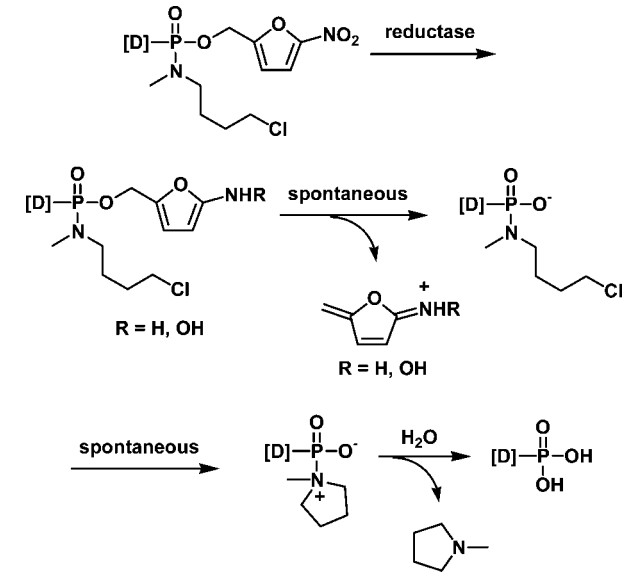


Figure 34. Activation of nitrofuranylmethyl amidate prodrugs.

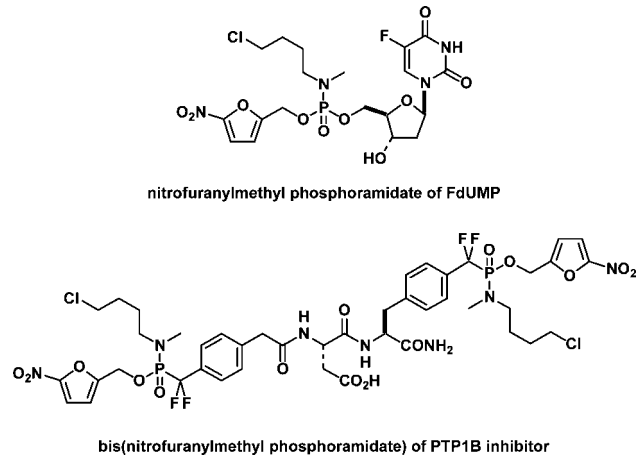


Figure 35. Nitrofuranylmethyl phosphoramidate prodrugs of FdUMP and a PTP1B inhibitor.

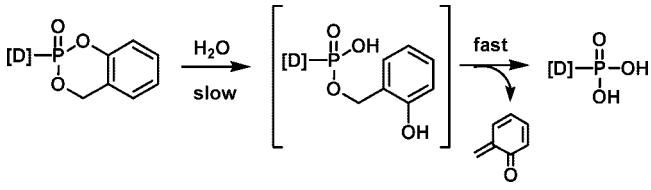


Figure 36. Activation of cycloSal prodrugs.

optimization of the degradation rate is difficult, since increasing the rate of release results in greater degradation prior to reaching the site of action, whereas decreasing it typically leads to clearance of the prodrug prior to release of the active drug. In addition, it may be difficult to achieve sufficient solid-state stability for drug product manufacturing and a reasonable shelf life. Most abundant among these approaches is the "cycloSal" prodrug advanced by Meier et al.[168] The first step in its cleavage involves a chemical hydrolysis that is highly sensitive to pH, after which the *o*-hydroxybenzyl intermediate rapidly fragments to produce the free phosph(on)ate (Figure 36).

The utility of cycloSal prodrugs to deliver the nucleoside monophosphate in cellular systems has been demonstrated with d4T (Figure 37)[169] and ddA,[170] as well as other nucleosides.[171] A second-generation "lock-in" approach has been described, in which

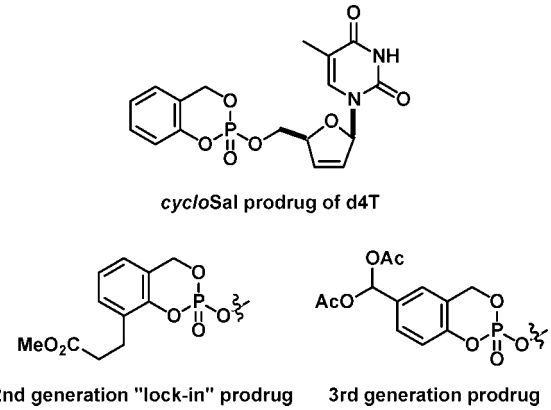


Figure 37. CycloSal prodrugs of d4T.

an esterase-cleavable group is appended to the phenyl ring in order to increase the intracellular concentration,[172] although these derivatives led to delayed drug delivery because the charged intermediate displayed greater chemical stability. A third-generation approach designed to enhance the rate of degradation following the initial esterase cleavage step has also been reported.[173] Application of the cycloSal approach to the phosphonic acid adefovir has also been described.[174] No examples of in vivo administration of cycloSal prodrugs have appeared.

Comparison of Prodrug Classes

Despite promising in vitro and in some cases in vivo studies, only a few of the prodrug classes described in the previous sections have been advanced into clinical studies. Given the effort and expense required, most agents only proceed into such studies after assembly of a convincing preclinical data package showing adequate stability for formulation and storage, a rate of prodrug cleavage sufficient to afford systemic exposure of the active drug in animal models and yet slow enough to survive enzymes present in the GI tract (for oral drugs), adequate safety with respect to prodrug byproducts, and the ability to synthesize substantial quantities in a reproducible manner. Of the various classes of phosph(on)ate prodrugs examined in this Perspective, only six have been represented in agents advanced to human clinical trials: acyloxyalkyl esters, aryl esters, aryl amidates, diamides, HepDirect esters, and lipid esters (Figure 38, Table 2). Of these six classes, all but the aryl esters currently are being actively developed or have advanced all the way to the market. A brief discussion of the various prodrug classes with respect to the key attributes for prodrug success is in order.

Chemical Stability. Stability is a key attribute of prodrugs. Since all classes represent esters and amides of phosphoric or phosphonic acids, the primary degradation pathway of concern is hydrolysis. Within each class, both the electronic environment around the phosph(on)ate as well as substitution on the prodrug moiety may have a profound effect on the stability of the prodrug. Therefore, in the course of prodrug optimization, stability should be assessed in parallel with biological properties and may have great influence on the selection of a development candidate. The definition of "sufficient" stability (in aqueous solution) will vary depending upon the physical form of the API (crystalline or amorphous) as well as the nature of the formulation (solution, suspension, or solid dosage form). In general, greater stability to aqueous hydrolysis is preferred because it affords greater flexibility in formulation development and storage of the drug product, whereas stability issues can limit formulation options, require more exotic (and expensive) formulations, and/or require cold storage.

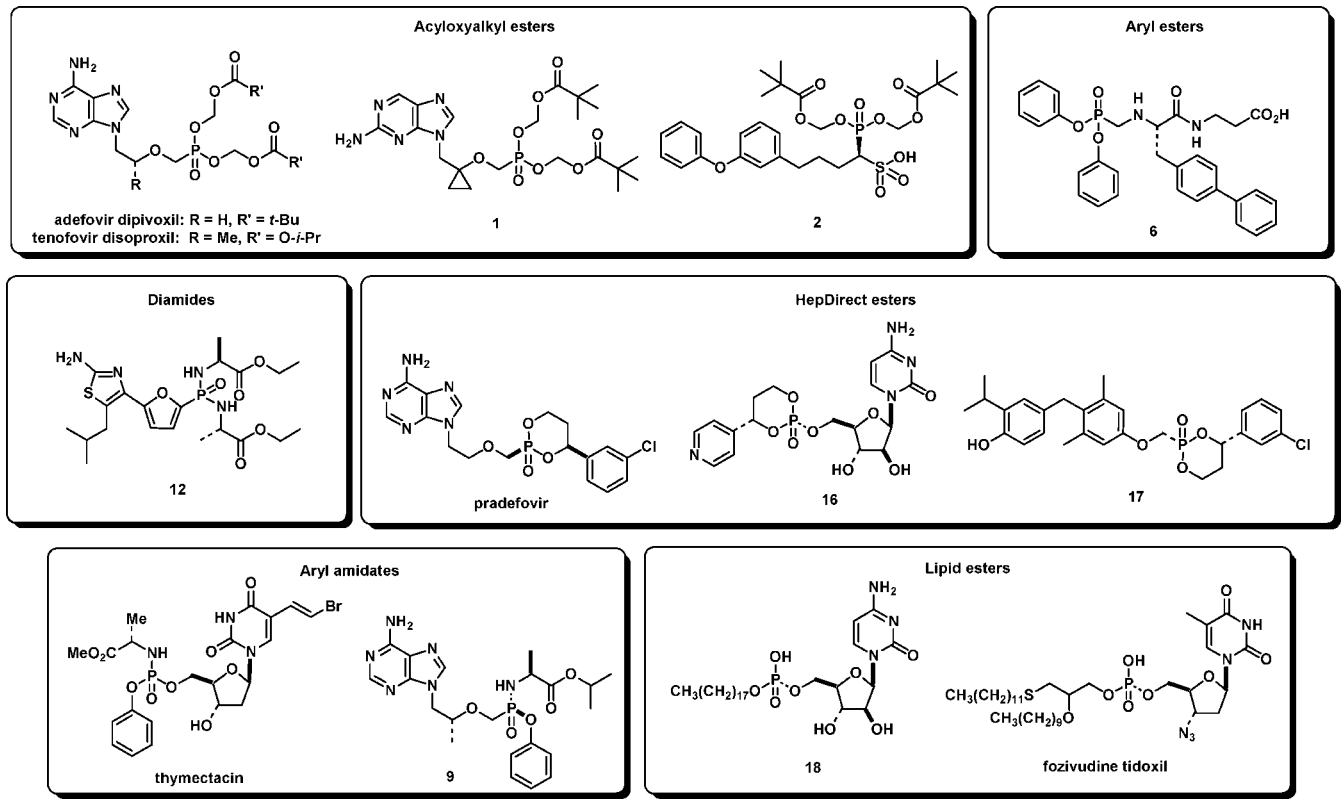
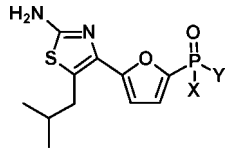


Figure 38. Prodrugs advanced into human clinical trials.

Table 1. Aqueous Stability, Esterase Conversion Rate and Oral Bioavailability of Prodrugs of **11**[121]



11: X, Y = OH

X	Y	t_{90}[a] (h)	ECR[b] ((nmol/min)/mg)	F[c] (%)
O-POM	O-POM	<4	>300	11
O-PAB	O-PAB	<4	>300	8
SATE	SATE	8	>300	ND
OPh	Ala-OEt	14	55	12
Gly-OEt	Gly-OEt	36	43	26
Ala-OEt	Ala-OEt	>48	11	22
Ala(Me)-OEt	Ala(Me)-OEt	>48	8	47

[a] Time to reach 90% of starting concentration at 37 °C in 100 mM potassium phosphate (pH 7). [b] ECR, esterase conversion rate for prodrugs incubated with rat liver S9 (2 mg/mL) at 37 °C. [c] Oral bioavailability determined by measuring urinary excretion of **11** following oral administration of the prodrug vs iv administration of **11**.

Reports containing data on aqueous stability of prodrugs are scant, and even more rare are reports comparing stability of different prodrug types under identical conditions. A notable exception is a very recent article describing the discovery of the FBPase inhibitor **11**, in which several classes of prodrugs were prepared and evaluated (Table 1).[121] At pH 7 and 37 °C, the bis(POM) and bis(*p*-acetoxybenzyl) prodrugs degraded rapidly, with a t_{90} (time to reach 90% of starting concentration) of less than 4 h. The bis(SATE) and aryl phosphonamidate prodrugs showed moderate stability (t_{90} of 8 and 14 h, respectively). Most stable of all were the diamide prodrugs derived from ethyl esters of glycine, alanine, and α-methylalanine, all of which displayed a t_{90} of over 24 h.

Table 2. Key Attributes of Prodrug Classes

prodrug class	taken to humans	enzyme(s) responsible	byproduct
acyloxyalkyl esters	yes	esterase	formaldehyde
SATE esters	no	esterase	episulfide
aryl esters	yes	unknown	phenol
benzyl esters (*p*-acetoxy)	no	esterase	quinone methide
benzyl esters (*p*-methoxy)	no	cytochrome P_{450}	quinone methide
aryl amidates	yes	esterase, amidase	phenol
diamides	yes	esterase, amidase	amino acid
HepDirect esters	yes	cytochrome P_{450}	aryl vinyl ketone
lipid esters	yes	phosphodiesterase	lipid
nitrofuranylmethyl amidates	no	reductase	furanyl methide
cycloSal esters	no	nonenzymatic	quinone methide

Within acyloxyalkyl prodrugs, the degradation kinetics of the bis(isopropyloxycarbonyloxymethyl) prodrugs of PMEA and PMPA (tenofovir disoproxil) have been described.[10] These studies concluded that chemical hydrolysis occurs predominately via P−O bond cleavage, with C−O bond cleavage being a minor pathway. Although this report is silent on the question of whether stability is a significant issue for these drugs, the existence of a patent application directed to a tablet formulation of adefovir pivoxil having improved stability suggests that indeed it is.[175]

The HepDirect prodrugs pradefovir and **16** show excellent stability, each with t_{90} at pH 7 (25 °C) of over 3 days.[125] Since **16** is formulated as a solution for intravenous administration, stability was a key aspect in its selection. Notably, the 4-pyridyl moiety affords stability superior to that of substituted phenyl analogues, with <1% degradation observed after 24 h at 37 °C.[136]

Lipid esters likely have no stability issues, since the phosph(on)ate group bears a negative charge at neutral pH and is thereby protected from nucleophilic attack by water.

Rate of Conversion. In general, the ideal prodrug will be perfectly stable until it reaches the desired site of activation, and then will be rapidly and quantitatively converted to the active drug. Like stability, rate of conversion is an attribute that can be easily measured using in vitro systems. However, unlike stability, it is difficult to define an optimal cleavage rate, since most of the cleaving enzymes are expressed in the GI tract. That is, while rapid cleavage is generally desirable, the rate must be slow enough for the majority of the prodrug to survive transit through the GI tract intact. In practice, the optimal cleavage rate is determined empirically by evaluating which prodrug achieves the highest systemic exposure to the active drug.

Like aqueous stability, there is little data available comparing rates of cleavage of various prodrug classes under the same conditions, with the notable exception of the prodrugs of the FBPase inhibitor **11** shown in Table 1. The bis(POM), bis(*p*-acetoxybenzyl) and bis(SATE) prodrugs are all converted by esterase exceedingly rapidly (>300 (nmol/min)/mg protein), suggesting that these will be most prone to premature cleavage in the GI tract. The aryl phosphonamidate and phosphonic diamide prodrugs are converted much more slowly (8–55 (nmol/min)/mg protein) and thus have a greater likelihood of surviving intact until they are absorbed. This may be a factor in their generally greater oral bioavailability (Table 1). HepDirect prodrugs, by comparison, are converted even more slowly. For example, pradefovir is converted at a rate of 0.5 (nmol/min)/mg protein in pooled human liver microsomes.[125] This feature ensures that the vast majority of the drug survives the GI tract to be absorbed, and yet cleavage in the liver is sufficiently fast to afford efficacious levels of the active drug. Aryl esters, despite the existence of successful examples, are generally thought to be too slowly cleaved to have widespread utility.

Byproduct Toxicity. Ideally, the cleavage byproducts of any prodrug would be completely innocuous. While this principle has been recognized for as long as prodrugs have been in existence, in practice it is difficult to achieve. Simple esters typically offer the greatest potential for nontoxic cleavage products, but in the case of phosph(on)ates they are far too stable toward enzymatic hydrolysis. In general, the design of "triggering groups" requires incorporation of moieties of unknown toxicity. In some cases, the toxicity is known and is of concern. If the nature of the toxicity is well understood and data are available relating exposure to effect, it may be possible to assess the risk by calculating the likely exposure to the byproduct following administration of a particular dose. Much more difficult to assess are the potential liabilities associated with reactive intermediates capable of forming covalent bonds with cellular macromolecules, which may lead to carcinogenicity or idiosyncratic toxicity. Great progress has been made in developing methods to assess byproduct-related covalent modification, but the relationship of these results to downstream risks is far less certain.[176]

Four factors play into the analysis of whether the risks associated with a particular byproduct are "acceptable" for drug development: the disease indication, the duration of treatment, the dose required for efficacy, and the in vivo compartment in which the byproduct is produced. Chronic medications for non-life-threatening diseases have a low tolerance for risk associated with byproducts, particularly those capable of covalently modifying macromolecules, although the concern diminishes for low-dose agents and those designed for short-term use. Life-threatening disease indications, such as cancer and the more dangerous infectious diseases, can accommodate some level of risk associated with a potentially toxic byproduct. Concerns related to an electrophilic byproduct may be alleviated if it is produced only in cells containing high levels of the endogenous nucleophile glutathione.

Acyloxyalkyl prodrugs generate an aldehyde and either a carboxylic acid (ester type) or an alcohol and carbon dioxide (carbonate type) (Table 2). For phosph(on)ates, the aldehyde is typically formaldehyde, since substitution on the acetal carbon greatly complicates synthesis and characterization by virtue of it being a stereogenic center. The generation of formaldehyde as a byproduct has long been of concern. This perception has not been substantiated by findings of prodrug toxicity, and two currently marketed phosphonate drugs, adefovir and tenofovir, contain a methylene unit that releases formaldehyde upon prodrug activation. SATE esters produce ethylene sulfide, a potent alkylating agent, as a byproduct. Consequently, it is not surprising that the utility of these prodrugs has been limited to being tool compounds for intracellular delivery of phosph(on)ates in cellular assays.

Aryl esters and aryl amidates produce phenol or a substituted phenol as a byproduct. Although phenol is generally considered to be highly toxic, it is not mutagenic, and therefore, the level of concern regarding its toxicity depends on the dose of the prodrug and the resulting dose of phenol that is released. HepDirect prodrugs produce an aryl vinyl ketone, a potent alkylating agent, which would be of potential concern were it not for the fact that cleavage is mediated by cytochrome P_{450} enzymes within hepatocytes, which contain a high concentration of the detoxifying nucleophile glutathione. As expected, the glutathione conjugate was detected in the serum of mice 6 h after intraperitoneal treatment with a high dose of the HepDirect prodrug pradefovir.[126] *p*-Methoxybenzyl esters are also cleaved oxidatively in the liver, in this case producing a quinone methide, which might also be trapped by glutathione (although this has not been studied). *p*-Acetoxybenzyl esters and cycloSal prodrugs produce a quinone methide but without the benefit of doing so in a tissue-specific manner, and thus, these prodrugs evoke a high level of concern relating to byproduct toxicity. Nitrofuranylmethyl amidates also release a highly electrophilic furan byproduct that is analogous to a quinone methide.

Diamides appear to pose the least risk from a byproduct perspective because they release a low molecular weight alcohol and a simple amino acid, neither of which raise toxicological concerns.

Stereochemical Issues. The phosphorus atom of phosph(on)ates is tetrahedral and thereby is capable of being a stereogenic center if the two appended prodrug groups are different. If the parent phosph(on)ate is achiral, this situation leads to two enantiomers (a racemate), whereas if the phosph(on)ate is chiral, the prodrug may exist as two diastereomers. From the perspective of drug development, it is far preferable to develop a single stereoisomer than either a racemate or a mixture of diastereomers. It is often the case that two enantiomers are cleaved at unequal rates by the enzyme responsible for cleavage, and in this case one of the enantiomers is by definition "preferred". Mixtures of diastereomers complicate matters further, since in addition to having different biological properties, they will be difficult to isolate in crystalline form, the ratio of diastereomers may be variable, and characterization of the product may be confounded.

Of the various classes discussed in this Perspective, most have two identical groups attached to the phosphorus atom, so there are no stereochemistry issues. The lipid esters are monoacids, and therefore, the phosphorus atom is not stereogenic by virtue of rapid exchange of the acid hydrogen atom between the two

oxygen atoms. Of the remaining classes having two different groups on phosphorus, the nitrofuranylmethyl amidates and cycloSal esters have not advanced far enough to have had to contend with the difficulties that stereoisomers present in drug development.

Of the two remaining classes, aryl amidates and HepDirect esters, the development of the former is clearly complicated by stereochemistry issues. The anticancer agent thymectacin is apparently being developed as a mixture of diastereomers,[107] whereas **9** was advanced as a single diastereomer, isolated on large scale using simulated moving bed (SMB) chromatography.[114,115] While HepDirect esters are capable of existing in two diastereomeric forms, the cis isomer (about the dioxaphosphinane ring) is generally activated much more readily than the trans isomer.[125] The cyclic nature of this diester confers significant differences in rate between the pathways for formation of the two isomers, which may be exploited for facile stereoselective synthesis.[127]

Tissue Targeting. The concept of delivering a drug to a particular tissue is extremely attractive because it would address two major reasons for failure of drug candidates, i.e., toxicity in an organ other than the one(s) where the drug exerts its desired effect and insufficient efficacy resulting from limited distribution to the target organ. The theoretical basis for use of prodrugs to achieve site-specific drug delivery was elegantly delineated by Tomlinson[177] and Stella.[178] Although simple in principle, tissue-targeting is rarely observed because most prodrugs are activated by enzymes that are widely distributed in tissues, including the first systemic compartment that is typically seen by the prodrug, the bloodstream. Demonstrating tissue targeting requires not only increased exposure in a particular tissue but also an increase in the *ratio* of exposure to the targeted tissue vs another tissue. The use of prodrugs for liver-targeted drug delivery has been reviewed.[179]

By far the most successful approach to tissue targeting via a phosph(on)ate prodrug is the HepDirect esters, a class specifically designed to achieve site-specific delivery to the liver in large part by virtue of their ability to undergo oxidative cleavage by cytochrome P_{450} in hepatocytes and intracellular trapping. The success of this approach has been clearly established in vivo. The PMEA prodrug pradefovir, in development for hepatitis B, achieves a 12-fold improvement in liver/kidney exposure ratio relative to the corresponding bis(POM) prodrug adefovir dipivoxil. The cytarabine prodrug **16**, in development for treatment of hepatocellular carcinoma, achieves a >28-fold increase in liver/bone marrow exposure ratio relative to administration of cytarabine. A third agent **17**, a liver-selective thyroid receptor agonist in development for hyperlipidemia, utilizes a HepDirect prodrug to enhance the liver distribution of a novel TR agonist and thereby unmasks the lipid lowering activity of this drug class without the dose-limiting side effects associated with non-liver-selective TR agonists.[138]

Lee et al. have described the observation of preferential distribution and accumulation of tenofovir in lymphatic tissue with **9**, an aryl amidate prodrug of tenofovir.[113] Following oral administration of **9** to beagle dogs, the ratio of the tenofovir AUC in peripheral blood mononuclear cells (PBMCs) vs plasma was >90, whereas following administration of tenofovir disoproxil fumarate (tenofovir DF), an acyloxyalkyl prodrug, the ratio was 4.7, representing about a 20-fold increase in PMBC to plasma ratio. In a radiolabeled tissue distribution study at 24 h postadministration, the concentration in lymph nodes was 5- to 15-fold higher with **9** relative to tenofovir DF. These researchers attributed this impressive tissue-specific delivery to substantial stability of **9** in plasma combined with rapid intracellular hydrolysis in PBMCs by cathepsin A, resulting in intracellular trapping and accumulation of the negatively charged phosphonate.[117] One may speculate that **15**, a diamide prodrug of a cytotoxic nucleotide recently reported from the same research group for treatment of lymphoid malignancies,[124] may capitalize on the same preferential distribution to lymphoid tissue.

The potential for tissue targeting has been investigated within the lipid ester class of prodrugs. As noted above, Hostetler et al. were initially interested in incorporating prodrugs of anti-HIV nucleosides into liposomes in order to enhance uptake into lymph nodes, spleen, and other HIV target tissues.[153] In vivo studies in a woodchuck model of hepatitis B infection were conducted with a liposomal formulation of 1,2-dipalmitoylphosphatidyldideoxyguanosine (DPP-ddG), which demonstrated improved efficacy relative to an equimolar dose of ddG.[180] However, while these and other studies showed enhanced exposure and/or efficacy, no evidence of enhanced therapeutic index or tissue targeting was provided.

Conclusions

The field of prodrugs for phosph(on)ates has evolved considerably in the past decade. There are now several approaches available to the researcher who finds that a phosphonate moiety achieves the right balance of properties in the lead series, yet needs a prodrug for oral delivery. The overall best choice in this situation, taking into account stability, byproduct toxicity, avoidance of stereoisomers, and validation in humans, appears to be the diamide class. Given its relatively recent appearance, there are few examples to date, but it is expected that phosphonic diamides will play an increasingly important role as phosphonate prodrugs going forward. For drugs given at low to moderate doses, where risks associated with the byproduct formaldehyde are low, acyloxyalkyl prodrugs will continue to find application. If selective targeting to the liver is desired, HepDirect prodrugs are the clear choice, whereas aryl amidates (and possibly diamides) offer the potential for targeting to lymphatic tissue. The increasing availability of prodrug options should encourage the practicing medicinal chemist to explore phosphonic acids in the course of lead development.

Biographies

Scott J. Hecker received his B.A. in Chemistry from Wesleyan University in 1980 and his Ph.D. in Organic Chemistry from the University of California, Berkeley, under Professor Clayton Heathcock in 1985. He then spent 8 years at Pfizer (Groton) in antibacterial discovery. In 1993 he joined Microcide Pharmaceuticals, where over a 10 year period he pursued discovery of novel anti-infectives, particularly cephalosporins, while assuming responsibility for Natural Products Research, Chemical Development and Intellectual Property. In 2003, he joined Metabasis Therapeutics as Executive Director of Chemistry, where he is responsible for the Medicinal and Process Chemistry groups. His scientific interests include synthetic methodology, prodrug design, and physicochemical properties of drugs and druglike molecules. He has authored over 35 publications and is a coinventor on 23 U.S. patents.

Mark D. Erion received his B.Sc. (Hons) in Mathematics and Chemistry from the University of Oregon in 1979 and his Ph.D. in Organic Chemistry from Cornell University under Professor John McMurry in 1984. Following an NIH postdoctoral fellowship in enzyme kinetics and molecular biology under Professor Christopher Walsh at Massachusetts Institute of Technology, he joined CIBA-Geigy (NJ), where he led projects in cardiovascular and inflammation, ultimately becoming group leader of protein engineering (Switzerland). In 1991, he joined Gensia Pharmaceuticals with

responsibility for Chemistry and Biochemistry. In 1997, he cofounded Metabasis Therapeutics where he is Chief Scientific Officer. His scientific interests include computer-assisted drug design, drug targeting, prodrug design, liver biology, and metabolic disease drug discovery. He has authored over 105 publications and is a coinventor on 35 U.S. patents.

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